

Evaluation of the Nephroprotective Effects of Methanolic Extract of *Hygrophila Auriculata* (Neermulli Plant)

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ABSTRACT

Nephrotoxicity remains a major clinical concern associated with exposure to drugs, environmental pollutants, and oxidative stress-inducing agents, leading to progressive renal impairment. The present study aimed to Evaluate the Renoprotective potential of Neermulli extract (*Hygrophila auriculata*)against Nephrotoxicant-induced cellular damage in vero cells. The Methanolic extract of Neermulli Plant was prepared and subjected to preliminary phytochemical screening to identify bioactive constituents. Medicinal plants have been extensively investigated for their therapeutic potential against renal disorders due to their rich content of bioactive phytochemicals such as flavonoids, phenolics, alkaloids, and terpenoids. Vero cells were cultured under standard conditions and exposed to a known Nephrotoxicant to induce oxidative stress-mediated cytotoxicity. The protective efficacy of the extract was assessed through cell viability (MTT assay), Estimation of antioxidant enzymes including superoxide dismutase (SOD) and Catalase (CAT), and measurement of lipid peroxidation (LPO) levels.Numerous studies have reported the Renoprotective effects of plant extracts against Nephrotoxicant-induced oxidative damage through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms.Nephrotoxicity refers to renal dysfunction caused by exposure to drugs, chemicals, or environmental pollutants that adversely affect kidney structure and function. The observed Renoprotective activity may be attributed to the antioxidant and free radical scavenging properties of its phytoconstituents. Neermulli extract exhibits significant protective effects against Nephrotoxicant-induced cellular injury in Vero cells, suggesting its potential as a natural therapeutic agent for preventing oxidative stress-mediated renal damage.

KEYWORDS: Neermulli, Nephrotoxicant, Antioxidant enzymes, Free radical

INTRODUCTION



Fig.1: Neermulli Plant

Hygrophila auriculata commonly known as Neermulli and it is a well-recognized medicinal plant belonging to the family Acanthaceae. The plant is widely distributed in tropical and subtropical regions

of India, Sri Lanka, and Southeast Asia, particularly in marshy lands, riverbanks, and wet agricultural fields. In traditional systems of medicine, especially *Ayurveda*, *Siddha* and folk medicine, *Hygrophila auriculata* has been extensively used for the management of various ailments related to the urinary, hepatic, and reproductive systems. The medicinal importance of *Hygrophila auriculata* is attributed to its rich phytochemical composition, which includes flavonoids, phenolic compounds, alkaloids, steroids, terpenoids, and glycosides. These bioactive constituents are known to possess antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, and diuretic properties. Traditionally, different parts of the plant, including leaves, roots, seeds, and stems, have been used in herbal formulations for treating kidney disorders, urinary tract infections, edema, and inflammatory conditions.

SCIENTIFIC CLASSIFICATION



Fig 2: *Hygrophila auriculata*

Kingdom: Plantae

Division/Phylum: Tracheophyta (Angiosperms)

Class: Magnoliopsida (Dicots)

Order: Lamiales (formerly often listed as Scrophulariales)

Family: Acanthaceae

Genus: *Hygrophila*

Species: *Hygrophila auriculata* (Schumach.) Heine

Other name : *Asteracantha longifolia*

TRADITIONAL USAGE OF NEERMULLI

The seeds and root are deeply integrated into Ayurvedic practices to treat conditions associated with Kapha and Vata doshas. Kidneys are vital organs responsible for maintaining physiological homeostasis through the regulation of electrolyte balance, acid–base equilibrium, blood pressure, and the excretion of metabolic waste products. Due to their high blood perfusion rate and active involvement in xenobiotic metabolism, renal tissues—particularly tubular epithelial cells—are highly vulnerable to toxic injury. Exposure to nephrotoxic agents such as chemotherapeutic drugs, antibiotics, environmental pollutants, and oxidative stress–inducing compounds often results in acute kidney injury (AKI) and may progress to chronic kidney disease (CKD) if left untreated (Lopez-Novoa et al., 2011; Perazella, 2018). Nephrotoxicity is a major limitation in clinical pharmacotherapy and environmental toxicology. Several nephrotoxins, including cisplatin, gentamicin, heavy metals, and reactive oxygen species (ROS)–generating chemicals, induce renal injury primarily through oxidative stress, mitochondrial dysfunction, inflammation, and apoptosis in renal tubular epithelial cells (Paller, 1998; Ramesh & Reeves, 2004). Excessive ROS production overwhelms the endogenous antioxidant defense system, leading to lipid peroxidation, protein oxidation, DNA damage, and eventual loss of cellular integrity (Valko et al., 2007).

In recent years, there has been growing interest in the use of medicinal plants and herbal formulations as alternative or complementary therapies for kidney-related disorders. Plant-derived bioactive compounds such as flavonoids, phenolics, alkaloids, tannins, and terpenoids have demonstrated strong antioxidant, anti-inflammatory, and cytoprotective properties, making them promising candidates for renal protection (Ajith et al., 2007; Olayinka et al., 2015).

MATERIALS AND METHODS

COLLECTION OF PLANT MATERIAL

Fresh and healthy plant material of *Hygrophila Auriculata* was collected from a local region during the appropriate growing season. The plant was authenticated by a qualified taxonomist, and a voucher specimen was deposited in the herbarium for future reference. The collected plant material was washed thoroughly with running tap water followed by distilled water to remove soil and debris and then shade-dried at room temperature for 10–15 days. The dried material was pulverized into a fine powder using a mechanical grinder and stored in an airtight container for further use.

PREPARATION OF PLANT EXTRACT

The powdered plant material was subjected to extraction using hydroalcoholic solvent by maceration. Briefly, a 50g of powdered material was soaked in Methanol and kept under continuous shaking for 48h at room temperature. The extract was filtered using Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator. The concentrated extract was dried, weighed to calculate percentage yield, and stored at 4 °C until further analysis. For cell culture studies, stock solutions were prepared using sterile DMSO and filtered through a 0.22 µm membrane filter.

PHYTOCHEMICAL ANALYSIS

Qualitative Phytochemical analysis

Phytochemical analysis were carried out with standard modified protocols for identification of presence and active phytoconstituents.

Test for Alkaloids

100 mg of extract was dissolved in 5 ml of 1% Hydrochloric acid and filtered. The filtrate was treated with 2 to 3 drops of Dragendorff's reagent, Formation of orange-red precipitate indicates the presence of Alkaloids.

Mayer's test for Alkaloids: To 1 ml of filtrate add few drops of Mayer's reagent appearance of yellowish white precipitate indicates the presence of alkaloids.

Test for Flavonoids

The plant extracts were dissolved in methanol by mild heating, to each of the extract small pieces of magnesium ribbon was added, followed by the addition of a few drops of concentrated hydrochloric acid. The change of colour from orange, pink, red to purple indicates the presence of flavonoids.

Test for Tannins

200 mg of each plant extract was dissolved in about 10 ml of distilled water and then filtered. Two ml of each extract was taken in separate test tube. Few drops of 1% alcoholic ferric chloride solution was added to test tubes and the occurrence of blue green precipitate indicates the presence of tannins.

Test for Terpenoids

500 mg of extract was dissolved in ethanol equal volume of dissolved extract and acetic anhydride were taken in a clean test tube. Few drops of concentrated sulphuric acid was added to the tubes. The colour change from pink to violet showed the presence terpenoids (or) formation of blue green ring indicates the presence of terpenoids.

Test for Saponins

One gram of each extract was shaken vigorously with 3 to 5 ml of distilled water persistent foam for 10 minutes confirm is the presence of saponins.

Cardiac glycosides (Keller-Killani test)

Three ml of each plant extract was treated with 2 ml of glacial acetic acid and few drops of 1% ferric chloride solution, 1 ml of concentrated sulphuric acid were added. Formation of brown ring at the interface shows the presence of cardiac glycosides.

Test for Phenols

A small of methanolic extract was taken with 1 ml of water in a test tube and 1 to 2 drops of (FeCl₃) was added. A blue, green, red or purple is a positive test.

THIN LAYER CHROMATOGRAPHY

Principle

Thin layer chromatography (TLC) is a type of planar chromatography. It is a convenient method for separation of many compounds based on polarity, binding tightly to the adsorbent and migrating less than others. (Stahl, 1969) TLC is a chromatographic technique which is applied for the separation of a mixture of compounds. The thin layer chromatography technique was explained and performed by the method of Lawrence et al., 2015; Abubaker et al., 2016; Stahl and Kaldewey, 1961.

Reagents required

TLC (Merck), all solvents used were of analytical grade. Procedure Preparation of sample

Solvent system (Mobile phase)

The different solvent systems were developed to run the TLC. The solvent system was made from methanol: chloroform in the ratio of 1:1.2 (v/v) for methanol extracts.

Preparation of TLC plate

The sterile silica coated plates with the size range of 2cm x 6cm were prepared and the bottom of the sheet is marked with 1 cm margin for the application of sample. The extract was spotted on the sheets separately with the help of capillary tubes and they were allowed to dry before introducing them in to the running chamber where the respective mobile phase is present. The glass jars marked with specific label are filled with respective mobile phase upto 1cm height from the bottom and the chambers were closed to prevent the evaporation of solvents.

Development of TLC plate

The TLC plates were visualized for band formation with a portable UV lamp and the R_f values were calculated by measuring the distance moved by the solvent and distance moved by solute using a scale. Calculation R_f value can be calculate using the formula

$$R_f = \frac{\text{Distance moved by spot front}}{\text{Distance moved by compounds}}$$

Cell Culture Procedure

Normal renal epithelial cells (Vero) were obtained from a certified cell repository. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37 °C in a humidified incubator with 5% CO₂. The culture medium was replaced every 2–3 days, and cells were subcultured upon reaching 80–90% confluence using trypsin-EDTA.

CELL VIABILITY ASSAY

MTT ASSAY

The Vero cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM™; Gibco®, Life Technologies™, USA) supplemented with 1% (v/v) antibiotics streptomycin (100 mg/mL) and penicillin (100 UI/mL) (Cultilab, São Paulo, Brazil), and 10% (v/v) heat-inactivated bovine fetal serum (Cultilab, São Paulo, Brazil) at 37 °C and 5% CO₂ humidified atmosphere.

$$\text{Calculation \% Of Cell viability} = \frac{\text{OD of Sample}}{\text{OD of Control}} \times 100$$

ANTIOXIDANT ASSAY

1. Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined based on the inhibition of nitrobluetetrazolium (NBT) reduction. Cell lysates were prepared by homogenizing treated cells in ice-cold phosphate buffer followed by centrifugation. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT reduction.

$$\% \text{ SOD Activity} = \frac{OD_{PC} - OD_{Sample}}{OD_{PC} - OD_{NC}}$$

2. Estimation of Catalase (CAT)

Catalase activity was measured by monitoring the decomposition of hydrogen peroxide. The reaction mixture consisted of phosphate buffer, hydrogen peroxide, and enzyme extract. Catalase activity was expressed as micromoles of hydrogen peroxide decomposed per minute per milligram of protein.

$$\% \text{ CAT Activity} = \frac{OD_{PC} - OD_{Sample}}{OD_{PC} - OD_{NC}}$$

RESULTS

Phytochemical Analysis

PHYTOCHEMICAL TESTS	RESULTS
Alkaloids	Moderate positive (++)
Saponins	Srong positive (+++)
Flavanoids	Moderate positive (++)
Tannis	Srong positive (+++)
Terpenoids	Not detected (-)
Phenol	Mild positive (+)
Glycosides	Not detected (-)

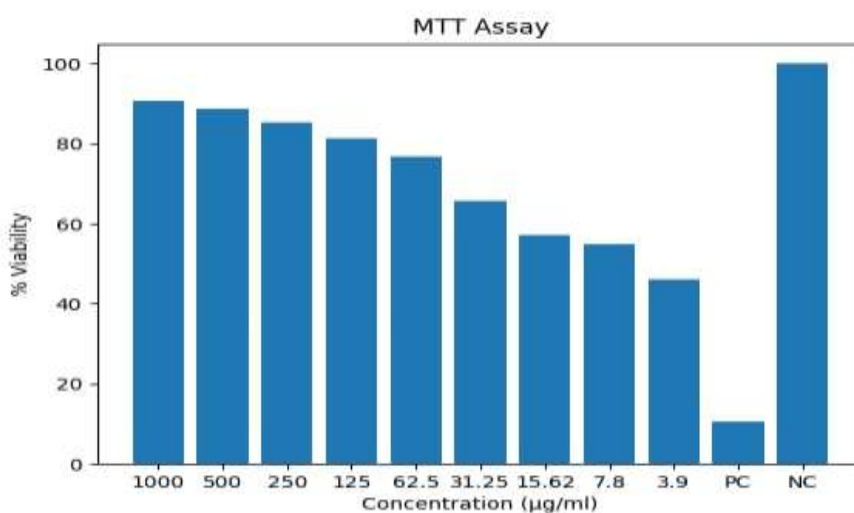
THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography of Neermuli methanolic extract was performed using methanol: chloroform (1:1.2 v/v) as the mobile phase.

Parameter	Observation
Distance travelled by solvent front	5.0 cm
Distance travelled by compound	2.8 cm (approx)
Rf value	0.56

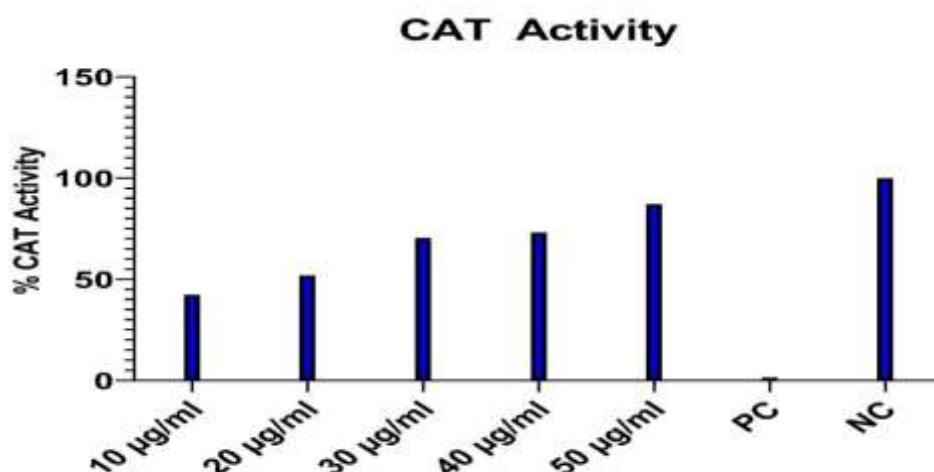
EVALUATION OF CELL VIABILITY USING MTT ASSAY

Concentration	Mean OD	Corrected OD	% Viability
Toxicant + 1000µg/ml	0.928667	0.91366667	90.9121061
Toxicant + 500µg/ml	0.905	0.881	87.6616915
Toxicant + 250µg/ml	0.871333	0.85233333	84.8092869
Toxicant + 125µg/ml	0.830667	0.80966667	80.5638474
Toxicant + 62.5µg/ml	0.784	0.758	75.4228856
Toxicant + 31.25µg/ml	0.670667	0.65366667	65.0414594
Toxicant + 15.62µg/ml	0.585333	0.56133333	55.854063
Toxicant + 7.8µg/ml	0.561	0.542	53.9303483
Toxicant + 3.9µg/ml	0.471667	0.44466667	44.2454395
Positive Control (Toxicant - Indoxylsulfate (250 µM)	0.107	0.088	8.75621891
Negative Control (Only Cells)	1.022	1.005	100

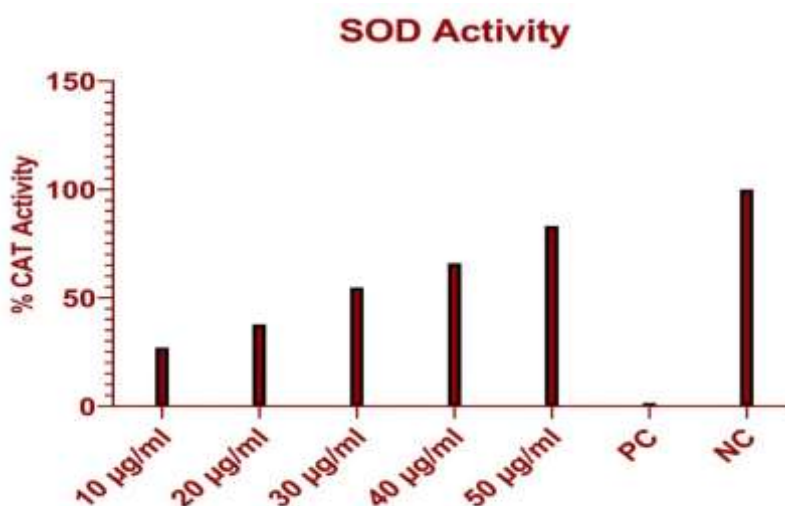


Graph : Cell Viability using (MTT Assay)

EVALUTION OF ANTIOXIDANT ENZYME ACTIVITY



Graph:CAT Activity



Graph:Superoxide dismutase (SOD) activity

DISCUSSION

The present study investigated the renoprotective potential of the methanolic extract of *Hygrophila auriculata* against indoxylsulfate-induced cytotoxicity in Vero cells, employing MTT assay for cell viability and antioxidant enzyme assays including superoxide dismutase (SOD) and catalase (CAT). In the current study, exposure to indoxylsulfate markedly reduced cell viability to approximately 8–9% compared to untreated control cells, confirming successful induction of severe oxidative cytotoxicity. Co-treatment with the methanolic extract of *Hygrophila auriculata* resulted in a clear and concentration-dependent restoration of cell viability. At lower concentrations, moderate recovery was observed, whereas higher concentrations restored viability to above 90%, approaching near-normal cellular metabolic function.

The phytochemical screening performed in this study revealed the presence of saponins and tannins in strong amounts, moderate levels of flavonoids and alkaloids, and mild presence of phenolic compounds, while terpenoids and glycosides were absent under the extraction conditions employed. The presence of flavonoids and phenolic compounds is particularly significant, as these molecules are well documented for their ability to scavenge superoxide radicals, hydroxyl radicals, and hydrogen peroxide. Therefore, the phytochemical composition identified in the present study provides a biochemical rationale for the

observed renoprotective effects. Oxidative stress plays a central role in indoxylsulfate-mediated nephrotoxicity. Superoxide radicals generated during toxicant exposure overwhelm the endogenous antioxidant defense system, leading to lipid peroxidation, protein oxidation, and DNA damage. At higher concentrations, SOD activity was restored to more than 80% of normal levels, indicating substantial neutralization of superoxide radicals. The results contribute to the growing body of evidence supporting the therapeutic potential of medicinal plants rich in phenolic and flavonoid compounds for the prevention and management of oxidative stress-related renal disorders.

CONCLUSION

The present study demonstrates that the methanolic extract of *Hygrophila auriculata* exhibits significant Renoprotective activity against indoxyl sulfate-induced cytotoxicity in Vero cells. However, treatment with the plant extract effectively restored cell viability in a clear concentration-dependent manner, indicating strong cytoprotective potential. The extract significantly enhanced superoxide dismutase (SOD) and catalase (CAT) activities, both of which were drastically reduced in the toxicant-treated group. Overall, the findings provide strong in vitro evidence supporting the Nephroprotective potential of *Hygrophila auriculata*. The study highlights its therapeutic relevance in preventing oxidative stress-related renal cellular damage.

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